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I, Janet Hope, BSc(Hons.), MIL., MITI., translator to Messrs. Taylor and Meyer of 20 Kingsmead Road, London, SW2 3JD, Great Britain, verify that I know well both the German and the English language, that I have prepared the attached English translation of 33 pages of a German Patent application in the German language with the title:

Neue für das deaD-Gen kodierende Nukleotidsequenzen

identified by the code number 000557 BT at the upper left of each page and that the attached English translation of this document is a true and correct translation of the document attached thereto to the best of my knowledge and belief.

I further declare that all statements made of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements are made with the knowledge that wilful false statements and the like are punishable by fine or imprisonment, or both, under 18 USC 1001, and that such false statements may jeopardize the validity of this document.

Signed:



Dated: 28 January 2004

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## Certificate of Priority for Filing of a Patent Application

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deaD gene

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**The attached papers are a true and accurate reproduction of the original  
documents for this patent application.**

Munich, 2nd August 2001

**On behalf of the President of the German  
Patent and Trade Mark Office**

*(signature)*

Jerofsky

**New nucleotide sequences which code for the dead gene**

The invention provides nucleotide sequences from coryneform bacteria which code for the dead gene and a process for the fermentative preparation of amino acids using bacteria in  
5 which the dead gene is attenuated.

**Prior art**

L-Amino acids, in particular L-lysine, are used in human medicine and in the pharmaceuticals industry, in the foodstuffs industry and very particularly in animal  
10 nutrition.

It is known that amino acids are prepared by fermentation from strains of coryneform bacteria, in particular *Corynebacterium glutamicum*. Because of their great importance, work is constantly being undertaken to improve  
15 the preparation processes. Improvements to the process can relate to fermentation measures, such as, for example, stirring and supply of oxygen, or the composition of the nutrient media, such as, for example, the sugar concentration during the fermentation, or the working up to  
20 the product form by, for example, ion exchange chromatography, or the intrinsic output properties of the microorganism itself.

Methods of mutagenesis, selection and mutant selection are used to improve the output properties of these  
25 microorganisms. Strains which are resistant to antimetabolites or are auxotrophic for metabolites of regulatory importance and which produce amino acids are obtained in this manner.

Methods of the recombinant DNA technique have also been  
30 employed for some years for improving the strain of *Corynebacterium* strains which produce L-amino acid, by

amplifying individual amino acid biosynthesis genes and investigating the effect on the amino acid production.

#### Object of the invention

The inventors had the object of providing new measures for improved fermentative preparation of amino acids.

#### Description of the invention

Where L-amino acids or amino acids are mentioned in the following, this means one or more amino acids, including their salts, chosen from the group consisting of L-asparagine, L-threonine, L-serine, L-glutamate, L-glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan and L-arginine. L-Lysine is particularly preferred.

When L-lysine or lysine are mentioned in the following, not only the bases but also the salts, such as e.g. lysine monohydrochloride or lysine sulfate, are meant by this.

The invention provides an isolated polynucleotide from coryneform bacteria, comprising a polynucleotide sequence which codes for the *deaD* gene, chosen from the group consisting of

- a) polynucleotide which is identical to the extent of at least 70% to a polynucleotide which codes for a polypeptide which comprises the amino acid sequence of SEQ ID No. 2,
- b) polynucleotide which codes for a polypeptide which comprises an amino acid sequence which is identical to the extent of at least 70% to the amino acid sequence of SEQ ID No. 2,
- c) polynucleotide which is complementary to the polynucleotides of a) or b), and

d) polynucleotide comprising at least 15 successive nucleotides of the polynucleotide sequence of a), b) or c),

the polypeptide preferably having the activity of DNA/RNA  
5 helicase.

The invention also provides the abovementioned polynucleotide, this preferably being a DNA which is capable of replication, comprising:

- (i) the nucleotide sequence, shown in SEQ ID No.1, or
- 10 (ii) at least one sequence which corresponds to sequence (i) within the range of the degeneration of the genetic code, or
- (iii) at least one sequence which hybridizes with the sequences complementary to sequences (i) or (ii),  
15 and optionally
- (iv) sense mutations of neutral function in (i).

The invention also provides:

a polynucleotide, in particular DNA, which is capable of replication and comprises the nucleotide sequence as  
20 shown in SEQ ID No.1;

a polynucleotide which codes for a polypeptide which comprises the amino acid sequence as shown in SEQ ID No. 2;

a vector containing parts of the polynucleotide according  
25 to the invention, but at least 15 successive nucleotides of the sequence claimed,

and coryneform bacteria in which the dead gene is attenuated, in particular by an insertion or deletion.

The invention also provides polynucleotides, which substantially comprise a polynucleotide sequence, which are obtainable by screening by means of hybridization of a corresponding gene library of a coryneform bacterium, which comprises the complete gene or parts thereof, with a probe which comprises the sequence of the polynucleotide according to the invention according to SEQ ID No.1 or a fragment thereof, and isolation of the polynucleotide sequence mentioned.

- 10 Polynucleotides which comprise the sequences according to the invention are suitable as hybridization probes for RNA, cDNA and DNA, in order to isolate, in the full length, nucleic acids or polynucleotides or genes which code for DNA/RNA helicase or to isolate those nucleic acids or  
15 polynucleotides or genes which have a high similarity with the sequence of the dead gene.

Polynucleotides which comprise the sequences according to the invention are furthermore suitable as primers with the aid of which DNA of genes which code for DNA/RNA helicase  
20 can be prepared by the polymerase chain reaction (PCR).

Such oligonucleotides which serve as probes or primers comprise at least 30, preferably at least 20, very particularly preferably at least 15 successive nucleotides. Oligonucleotides with a length of at least 40 or 50  
25 nucleotides are also suitable.

"Isolated" means separated out of its natural environment.

"Polynucleotide" in general relates to polyribonucleotides and polydeoxyribonucleotides, it being possible for these to be non-modified RNA or DNA or modified RNA or DNA.

- 30 The polynucleotides according to the invention include a polynucleotide according to SEQ ID No. 1 or a fragment prepared therefrom and also those which are at least 70%, preferably at least 80% and in particular at least 90% to

95% identical to the polynucleotide according to SEQ ID No. 1 or a fragment prepared therefrom.

"Polypeptides" are understood as meaning peptides or proteins which comprise two or more amino acids bonded via peptide bonds.

The polypeptides according to the invention include a polypeptide according to SEQ ID No. 2, in particular those with the biological activity of DNA/RNA helicase and also those which are at least 70%, preferably at least 80% and in particular at least 90% to 95% identical to the polypeptide according to SEQ ID No. 2 and have the activity mentioned.

The invention furthermore relates to a process for the fermentative preparation of amino acids chosen from the group consisting of L-asparagine, L-threonine, L-serine, L-glutamate, L-glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan and L-arginine using coryneform bacteria which in particular already produce amino acids and in which the nucleotide sequences which code for the dead gene are attenuated, in particular eliminated or expressed at a low level.

The term "attenuation" in this connection describes the reduction or elimination of the intracellular activity of one or more enzymes (proteins) in a microorganism which are coded by the corresponding DNA, for example by using a weak promoter or using a gene or allele which codes for a corresponding enzyme with a low activity or inactivates the corresponding gene or enzyme (protein), and optionally combining these measures.

The microorganisms provided by the present invention can prepare amino acids from glucose, sucrose, lactose, fructose, maltose, molasses, starch, cellulose or from

glycerol and ethanol. They can be representatives of coryneform bacteria, in particular of the genus *Corynebacterium*. Of the genus *Corynebacterium*, there may be mentioned in particular the species *Corynebacterium*  
5 *glutamicum*, which is known among experts for its ability to produce L-amino acids.

Suitable strains of the genus *Corynebacterium*, in particular of the species *Corynebacterium glutamicum* (*C. glutamicum*), are in particular the known wild-type strains

- 10 *Corynebacterium glutamicum* ATCC13032
- Corynebacterium acetoglutamicum* ATCC15806
- Corynebacterium acetoacidophilum* ATCC13870
- Corynebacterium melassecola* ATCC17965
- Corynebacterium thermoaminogenes* FERM BP-1539
- 15 *Brevibacterium flavum* ATCC14067
- Brevibacterium lactofermentum* ATCC13869 and
- Brevibacterium divaricatum* ATCC14020

and L-amino acid-producing mutants or strains prepared therefrom.

- 20 The new *deaD* gene from *C. glutamicum* which codes for the enzyme DNA/RNA helicase has been isolated.

- To isolate the *deaD* gene or also other genes of *C. glutamicum*, a gene library of this microorganism is first set up in *Escherichia coli* (*E. coli*). The setting up of  
25 gene libraries is described in generally known textbooks and handbooks. The textbook by Winnacker: *Gene und Klone, Eine Einführung in die Gentechnologie* (Verlag Chemie, Weinheim, Germany, 1990), or the handbook by Sambrook et al.: *Molecular Cloning, A Laboratory Manual* (Cold Spring  
30 Harbor Laboratory Press, 1989) may be mentioned as an example. A well-known gene library is that of the *E. coli* K-12 strain W3110 set up in  $\lambda$  vectors by Kohara et al. (Cell 50, 495 -508 (1987)). Bathe et al. (Molecular and



General Genetics, 252:255-265, 1996) describe a gene library of *C. glutamicum* ATCC13032, which was set up with the aid of the cosmid vector SuperCos I (Wahl et al., 1987, Proceedings of the National Academy of Sciences USA, 84:2160-2164) in the *E. coli* K-12 strain NM554 (Raleigh et al., 1988, Nucleic Acids Research 16:1563-1575).

Börmann et al. (Molecular Microbiology 6(3), 317-326) (1992) in turn describe a gene library of *C. glutamicum* ATCC13032 using the cosmid pH79 (Hohn and Collins, 1980, Gene 11, 291-298).

To prepare a gene library of *C. glutamicum* in *E. coli* it is also possible to use plasmids such as pBR322 (Bolivar, 1979, Life Sciences, 25, 807-818) or pUC9 (Vieira et al., 1982, Gene, 19:259-268). Suitable hosts are, in particular, those *E. coli* strains which are restriction- and recombination-defective, such as, for example, the strain DH5 $\alpha$ mc<sup>r</sup>, which has been described by Grant et al. (Proceedings of the National Academy of Sciences USA, 87 (1990) 4645-4649). The long DNA fragments cloned with the aid of cosmids or other  $\lambda$  vectors can then in turn be subcloned and subsequently sequenced in the usual vectors which are suitable for DNA sequencing, such as is described e. g. by Sanger et al. (Proceedings of the National Academy of Sciences of the United States of America, 74:5463-5467, 1977).

The resulting DNA sequences can then be investigated with known algorithms or sequence analysis programs, such as e.g. that of Staden (Nucleic Acids Research 14, 217-232(1986)), that of Marck (Nucleic Acids Research 16, 1829-1836 (1988)) or the GCG program of Butler (Methods of Biochemical Analysis 39, 74-97 (1998)).

The new DNA sequence of *C. glutamicum* which codes for the *deaD* gene and which, as SEQ ID No. 1, is a constituent of the present invention has been found. The amino acid

sequence of the corresponding protein has furthermore been derived from the present DNA sequence by the methods described above. The resulting amino acid sequence of the *deaD* gene product is shown in SEQ ID No. 2:

5 Coding DNA sequences which result from SEQ ID No. 1 by the degeneracy of the genetic code are also a constituent of the invention. In the same way, DNA sequences which hybridize with SEQ ID No. 1 or parts of SEQ ID No. 1 are a constituent of the invention. Conservative amino acid  
10 exchanges, such as e.g. exchange of glycine for alanine or of aspartic acid for glutamic acid in proteins, are furthermore known among experts as "sense mutations" which do not lead to a fundamental change in the activity of the protein, i.e. are of neutral function. It is furthermore  
15 known that changes on the N and/or C terminus of a protein cannot substantially impair or can even stabilize the function thereof. Information in this context can be found by the expert, inter alia, in Ben-Bassat et al. (Journal of Bacteriology 169:751-757 (1987)), in O'Regan et al. (Gene  
20 77:237-251 (1989)), in Sahin-Toth et al. (Protein Sciences 3:240-247 (1994)), in Hochuli et al. (Bio/Technology 6:1321-1325 (1988)) and in known textbooks of genetics and molecular biology. Amino acid sequences which result in a corresponding manner from SEQ ID No. 2 are also a  
25 constituent of the invention.

In the same way, DNA sequences which hybridize with SEQ ID No. 1 or parts of SEQ ID No. 1 are a constituent of the invention. Finally, DNA sequences which are prepared by the polymerase chain reaction (PCR) using primers which result  
30 from SEQ ID No. 1 are a constituent of the invention. Such oligonucleotides typically have a length of at least 15 nucleotides.

Instructions for identifying DNA sequences by means of hybridization can be found by the expert, inter alia, in  
35 the handbook "The DIG System Users Guide for Filter

Hybridization" from Boehringer Mannheim GmbH (Mannheim, Germany, 1993) and in Liebl et al. (International Journal of Systematic Bacteriology 41: 255-260 (1991)). The hybridization takes place under stringent conditions, that is to say only hybrids in which the probe and target sequence, i. e. the polynucleotides treated with the probe, are at least 70% identical are formed. It is known that the stringency of the hybridization, including the washing steps, is influenced or determined by varying the buffer composition, the temperature and the salt concentration. The hybridization reaction is preferably carried out under a relatively low stringency compared with the washing steps (Hybaid Hybridisation Guide, Hybaid Limited, Teddington, UK, 1996).

A 5x SSC buffer at a temperature of approx. 50°C - 68°C, for example, can be employed for the hybridization reaction. Probes can also hybridize here with polynucleotides which are less than 70% identical to the sequence of the probe. Such hybrids are less stable and are removed by washing under stringent conditions. This can be achieved, for example, by lowering the salt concentration to 2x SSC and optionally subsequently 0.5x SSC (The DIG System User's Guide for Filter Hybridisation, Boehringer Mannheim, Mannheim, Germany, 1995) a temperature of approx. 50°C - 68°C being established. It is optionally possible to lower the salt concentration to 0.1x SSC. Polynucleotide fragments which are, for example, at least 70% or at least 80% or at least 90% to 95% identical to the sequence of the probe employed can be isolated by increasing the hybridization temperature stepwise from 50°C to 68°C in steps of approx. 1 - 2°C. Further instructions on hybridization are obtainable on the market in the form of so-called kits (e.g. DIG Easy Hyb from Roche Diagnostics GmbH, Mannheim, Germany, Catalogue No. 1603558).

Instructions for amplification of DNA sequences with the aid of the polymerase chain reaction (PCR) can be found by the expert, inter alia, in the handbook by Gait: Oligonucleotide Synthesis: A Practical Approach (IRL Press, Oxford, UK, 1984) and in Newton and Graham: PCR (Spektrum Akademischer Verlag, Heidelberg, Germany, 1994).

It has been found that coryneform bacteria produce amino acids in an improved manner after attenuation of the dead gene.

10 To achieve an attenuation, either the expression of the dead gene or the catalytic properties of the enzyme protein can be reduced or eliminated. The two measures can optionally be combined.

15 The reduction in gene expression can take place by suitable culturing or by genetic modification (mutation) of the signal structures of gene expression. Signal structures of gene expression are, for example, repressor genes, activator genes, operators, promoters, attenuators, ribosome binding sites, the start codon and terminators.

20 The expert can find information on this e.g. in patent application WO 96/15246, in Boyd and Murphy (Journal of Bacteriology 170: 5949 (1988)), in Voskuil and Chambliss (Nucleic Acids Research 26: 3548 (1998)), in Jensen and Hammer (Biotechnology and Bioengineering 58: 191 (1998)),  
25 in Pátek et al. (Microbiology 142: 1297 (1996)), Vasicova et al. (Journal of Bacteriology 181: 6188 (1999)) and in known textbooks of genetics and molecular biology, such as e.g. the textbook by Knippers ("Molekulare Genetik", 6th edition, Georg Thieme Verlag, Stuttgart, Germany, 1995) or  
30 that by Winnacker ("Gene und Klone", VCH Verlagsgesellschaft, Weinheim, Germany, 1990).

Mutations which lead to a change or reduction in the catalytic properties of enzyme proteins are known from the prior art; examples which may be mentioned are the works by

Qiu and Goodman (Journal of Biological Chemistry 272: 8611-8617 (1997)), Sugimoto et al. (Bioscience Biotechnology and Biochemistry 61: 1760-1762 (1997)) and Möckel ("Die Threonindehydratase aus Corynebacterium glutamicum: Aufhebung der allosterischen Regulation und Struktur des Enzyms", Reports from the Jülich Research Centre, Jül-2906, ISSN09442952, Jülich, Germany, 1994). Summarizing descriptions can be found in known textbooks of genetics and molecular biology, such as e.g. that by Hagemann ("Allgemeine Genetik", Gustav Fischer Verlag, Stuttgart, 1986).

Possible mutations are transitions, transversions, insertions and deletions. Depending on the effect of the amino acid exchange on the enzyme activity, "missense mutations" or "nonsense mutations" are referred to. Insertions or deletions of at least one base pair (bp) in a gene lead to frame shift mutations, as a consequence of which incorrect amino acids are incorporated or translation is interrupted prematurely. Deletions of several codons typically lead to a complete loss of the enzyme activity. Instructions on generation of such mutations are prior art and can be found in known textbooks of genetics and molecular biology, such as e.g. the textbook by Knippers ("Molekulare Genetik", 6th edition, Georg Thieme Verlag, Stuttgart, Germany, 1995), that by Winnacker ("Gene und Klone", VCH Verlagsgesellschaft, Weinheim, Germany, 1990) or that by Hagemann ("Allgemeine Genetik", Gustav Fischer Verlag, Stuttgart, 1986).

A common method of mutating genes of *C. glutamicum* is the method of "gene disruption" and "gene replacement" described by Schwarzer and Pühler (Bio/Technology 9, 84-87 (1991)).

In the method of gene disruption a central part of the coding region of the gene of interest is cloned in a plasmid vector which can replicate in a host (typically *E.*

coli), but not in *C. glutamicum*. Possible vectors are, for example, pSUP301 (Simon et al., Bio/Technology 1, 784-791 (1983)), pK18mob or pK19mob (Schäfer et al., Gene 145, 69-73 (1994)), pK18mobsacB or pK19mobsacB (Jäger et al.,  
5 Journal of Bacteriology 174: 5462-65 (1992)), pGEM-T (Promega corporation, Madison, WI, USA), pCR2.1-TOPO (Shuman (1994). Journal of Biological Chemistry 269:32678-84; US Patent 5,487,993), pCR®Blunt (Invitrogen, Groningen, Holland; Bernard et al., Journal of Molecular  
10 Biology, 234: 534-541 (1993)) or pEM1 (Schrumpf et al, 1991, Journal of Bacteriology 173:4510-4516). The plasmid vector which contains the central part of the coding region of the gene is then transferred into the desired strain of *C. glutamicum* by conjugation or transformation. The method  
15 of conjugation is described, for example, by Schäfer et al. (Applied and Environmental Microbiology 60, 756-759 (1994)). Methods for transformation are described, for example, by Thierbach et al. (Applied Microbiology and Biotechnology 29, 356-362 (1988)), Dunican and Shivnan  
20 (Bio/Technology 7, 1067-1070 (1989)) and Tauch et al. (FEMS Microbiological Letters 123, 343-347 (1994)). After homologous recombination by means of a "cross-over" event, the coding region of the gene in question is interrupted by the vector sequence and two incomplete alleles are  
25 obtained, one lacking the 3' end and one lacking the 5' end. This method has been used, for example, by Fitzpatrick et al. (Applied Microbiology and Biotechnology 42, 575-580 (1994)) to eliminate the *recA* gene of *C. glutamicum*.

In the method of "gene replacement", a mutation, such as  
30 e.g. a deletion, insertion or base exchange, is established in vitro in the gene of interest. The allele prepared is in turn cloned in a vector which is not replicative for *C. glutamicum* and this is then transferred into the desired host of *C. glutamicum* by transformation or conjugation.  
35 After homologous recombination by means of a first "cross-over" event which effects integration and a suitable second

"cross-over" event which effects excision in the target gene or in the target sequence, the incorporation of the mutation or of the allele is achieved. This method was used, for example, by Peters-Wendisch et al. (Microbiology 5 144, 915 - 927 (1998)) to eliminate the pyc gene of *C. glutamicum* by a deletion.

A deletion, insertion or a base exchange can be incorporated into the *deadD* gene in this manner.

In addition, it may be advantageous for the production of 10 L-amino acids to enhance, in particular over-express, one or more enzymes of the particular biosynthesis pathway, of glycolysis, of anaplerosis, of the citric acid cycle, of the pentose phosphate cycle, of amino acid export and optionally regulatory proteins, in addition to the 15 attenuation of the *deadD* gene.

Thus, for the preparation of L-amino acids, in addition to the attenuation of the *deadD* gene at the same time one or more of the genes chosen from the group consisting of

- the *dapA* gene which codes for dihydrodipicolinate 20 synthase (EP-B 0 197 335),
- the *gap* gene which codes for glyceraldehyde 3-phosphate dehydrogenase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086),
- the *tpi* gene which codes for triose phosphate isomerase 25 (Eikmanns (1992), Journal of Bacteriology 174:6076-6086),
- the *pgk* gene which codes for 3-phosphoglycerate kinase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086),
- the *zwf* gene which codes for glucose 6-phosphate dehydrogenase (JP-A-09224661),
- 30 • the *pyc* gene which codes for pyruvate carboxylase (DE-A-198 31 609),

- the mgo gene which codes for malate-quinone oxidoreductase (Molenaar et al., European Journal of Biochemistry 254, 395-403 (1998)),
- 5 • the lysC gene which codes for a feed-back resistant aspartate kinase (Accession No.P26512),
- the lysE gene which codes for lysine export (DE-A-195 48 222),
- the hom gene which codes for homoserine dehydrogenase (EP-A 0131171),
- 10 • the ilvA gene which codes for threonine dehydratase (Möckel et al., Journal of Bacteriology (1992) 8065-8072)) or the ilvA(Fbr) allele which codes for a "feed back resistant" threonine dehydratase (Möckel et al., (1994) Molecular Microbiology 13: 833-842),
- 15 • the ilvBN gene which codes for acetohydroxy-acid synthase (EP-B 0356739),
- the ilvD gene which codes for dihydroxy-acid dehydratase (Sahm and Eggeling (1999) Applied and Environmental Microbiology 65: 1973-1979),
- 20 • the zwal gene which codes for the Zwal protein (DE: 19959328.0, DSM 13115)

can be enhanced, in particular over-expressed.

It may furthermore be advantageous for the production of amino acids, in addition to the attenuation of the dead  
25 gene, at the same time for one or more of the genes chosen from the group consisting of

- the pck gene which codes for phosphoenol pyruvate carboxykinase (DE 199 50 409.1, DSM 13047),



- the *pgi* gene which codes for glucose 6-phosphate isomerase (US 09/396,478, DSM 12969),
- the *poxB* gene which codes for pyruvate oxidase (DE:1995 1975.7, DSM 13114),
- 5 • the *zwa2* gene which codes for the Zwa2 protein (DE: 19959327.2, DSM 13113)

to be attenuated, in particular for the expression thereof to be reduced.

10 In addition to the attenuation of the *deaD* gene it may furthermore be advantageous for the production of amino acids to eliminate undesirable side reactions (Nakayama: "Breeding of Amino Acid Producing Microorganisms", in: Overproduction of Microbial Products, Krumphanzl, Sikyta, Vanek (eds.), Academic Press, London, UK, 1982).

15 The invention also provides the microorganisms prepared according to the invention, and these can be cultured continuously or discontinuously in the batch process (batch culture) or in the fed batch (feed process) or repeated fed batch process (repetitive feed process) for the purpose of  
20 production of L-amino acids. A summary of known culture methods is described in the textbook by Chmiel (Bioprozesstechnik 1. Einführung in die Bioverfahrenstechnik (Gustav Fischer Verlag, Stuttgart, 1991)) or in the textbook by Storhas (Bioreaktoren und  
25 periphere Einrichtungen (Vieweg Verlag, Braunschweig/Wiesbaden, 1994)).

The culture medium to be used must meet the requirements of the particular strains in a suitable manner. Descriptions of culture media for various microorganisms are contained  
30 in the handbook "Manual of Methods for General Bacteriology" of the American Society for Bacteriology (Washington D.C., USA, 1981).

Sugars and carbohydrates, such as e.g. glucose, sucrose, lactose, fructose, maltose, molasses, starch and cellulose, oils and fats, such as, for example, soya oil, sunflower oil, groundnut oil and coconut fat, fatty acids, such as, for example, palmitic acid, stearic acid and linoleic acid, alcohols, such as, for example, glycerol and ethanol, and organic acids, such as, for example, acetic acid, can be used as the source of carbon. These substances can be used individually or as a mixture.

Organic nitrogen-containing compounds, such as peptones, yeast extract, meat extract, malt extract, corn steep liquor, soya bean flour and urea, or inorganic compounds, such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate, can be used as the source of nitrogen. The sources of nitrogen can be used individually or as a mixture.

Phosphoric acid, potassium dihydrogen phosphate or dipotassium hydrogen phosphate or the corresponding sodium-containing salts can be used as the source of phosphorus.

The culture medium must furthermore comprise salts of metals, such as, for example, magnesium sulfate or iron sulfate, which are necessary for growth. Finally, essential growth substances, such as amino acids and vitamins, can be employed in addition to the abovementioned substances.

Suitable precursors can moreover be added to the culture medium. The starting substances mentioned can be added to the culture in the form of a single batch, or can be fed in during the culture in a suitable manner.

Basic compounds, such as sodium hydroxide, potassium hydroxide, ammonia or aqueous ammonia, or acid compounds, such as phosphoric acid or sulfuric acid, can be employed in a suitable manner to control the pH of the culture. Antifoams, such as, for example, fatty acid polyglycol esters, can be employed to control the development of foam. Suitable substances having a selective action, such as, for

example, antibiotics, can be added to the medium to maintain the stability of plasmids. To maintain aerobic conditions, oxygen or oxygen-containing gas mixtures, such as, for example, air, are introduced into the culture. The  
5 temperature of the culture is usually 20°C to 45°C, and preferably 25°C to 40°C. Culturing is continued until a maximum of the desired product has formed. This target is usually reached within 10 hours to 160 hours.

Methods for the determination of L-amino acids are known  
10 from the prior art. The analysis can thus be carried out, for example, as described by Spackman et al. (Analytical Chemistry, 30, (1958), 1190) by anion exchange chromatography with subsequent ninhydrin derivatization, or  
15 it can be carried out by reversed phase HPLC, for example as described by Lindroth et al. (Analytical Chemistry (1979) 51: 1167-1174).

The process according to the invention is used for fermentative preparation of amino acids.

The present invention is explained in more detail in the  
20 following with the aid of embodiment examples.

The isolation of plasmid DNA from Escherichia coli and all techniques of restriction, Klenow and alkaline phosphatase treatment were carried out by the method of Sambrook et al. (Molecular Cloning. A Laboratory Manual, 1989, Cold Spring  
25 Harbour Laboratory Press, Cold Spring Harbor, NY, USA). Methods for transformation of Escherichia coli are also described in this handbook.

The composition of the usual nutrient media, such as LB or TY medium, can also be found in the handbook by Sambrook et  
30 al.

Example 1

Preparation of a genomic cosmid gene library from *C. glutamicum* ATCC 13032

- Chromosomal DNA from *C. glutamicum* ATCC 13032 was isolated as described by Tauch et al. (1995, Plasmid 33:168-179) and partly cleaved with the restriction enzyme Sau3AI (Amersham Pharmacia, Freiburg, Germany, Product Description Sau3AI, Code no. 27-0913-02). The DNA fragments were dephosphorylated with shrimp alkaline phosphatase (Roche Molecular Biochemicals, Mannheim, Germany, Product Description SAP, Code no. 1758250). The DNA of the cosmid vector SuperCos1 (Wahl et al. (1987), Proceedings of the National Academy of Sciences, USA 84:2160-2164), obtained from Stratagene (La Jolla, USA, Product Description SuperCos1 Cosmid Vector Kit, Code no. 251301) was cleaved with the restriction enzyme XbaI (Amersham Pharmacia, Freiburg, Germany, Product Description XbaI, Code no. 27-0948-02) and likewise dephosphorylated with shrimp alkaline phosphatase.
- The cosmid DNA was then cleaved with the restriction enzyme BamHI (Amersham Pharmacia, Freiburg, Germany, Product Description BamHI, Code no. 27-0868-04). The cosmid DNA treated in this manner was mixed with the treated ATCC13032 DNA and the batch was treated with T4 DNA ligase (Amersham Pharmacia, Freiburg, Germany, Product Description T4-DNA-Ligase, Code no.27-0870-04). The ligation mixture was then packed in phages with the aid of Gigapack II XL Packing Extract (Stratagene, La Jolla, USA, Product Description Gigapack II XL Packing Extract, Code no. 200217).
- For infection of the *E. coli* strain NM554 (Raleigh et al. 1988, Nucleic Acid Res. 16:1563-1575) the cells were taken up in 10 mM MgSO<sub>4</sub> and mixed with an aliquot of the phage suspension. The infection and titering of the cosmid library were carried out as described by Sambrook et al.

(1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor), the cells being plated out on LB agar (Lennox, 1955, Virology, 1:190) + 100 µg/ml ampicillin. After incubation overnight at 37°C, recombinant individual clones  
5 were selected.

### Example 2

#### Isolation and sequencing of the dead gene

The cosmid DNA of an individual colony was isolated with the Qiaprep Spin Miniprep Kit (Product No. 27106, Qiagen, Hilden, Germany) in accordance with the manufacturer's  
10 instructions and partly cleaved with the restriction enzyme Sau3AI (Amersham Pharmacia, Freiburg, Germany, Product Description Sau3AI, Product No. 27-0913-02). The DNA fragments were dephosphorylated with shrimp alkaline  
15 phosphatase (Roche Molecular Biochemicals, Mannheim, Germany, Product Description SAP, Product No. 1758250). After separation by gel electrophoresis, the cosmid fragments in the size range of 1500 to 2000 bp were isolated with the QiaExII Gel Extraction Kit (Product No.  
20 20021, Qiagen, Hilden, Germany).

The DNA of the sequencing vector pZero-1, obtained from Invitrogen (Groningen, The Netherlands, Product Description Zero Background Cloning Kit, Product No. K2500-01) was cleaved with the restriction enzyme BamHI (Amersham  
25 Pharmacia, Freiburg, Germany, Product Description BamHI, Product No. 27-0868-04). The ligation of the cosmid fragments in the sequencing vector pZero-1 was carried out as described by Sambrook et al. (1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor), the DNA mixture  
30 being incubated overnight with T4 ligase (Pharmacia Biotech, Freiburg, Germany). This ligation mixture was then electroporated (Tauch et al. 1994, FEMS Microbiol. Letters, 123:343-7) into the E. coli strain DH5αmc<sup>r</sup> (Grant, 1990, Proceedings of the National Academy of Sciences, U.S.A.,

87:4645-4649). Letters, 123:343-7) and plated out on LB agar (Lennox, 1955, Virology, 1:190) with 50 µg/ml zeocin.

The plasmid preparation of the recombinant clones was carried out with the Biorobot 9600 (Product No. 900200, 5 Qiagen, Hilden, Germany). The sequencing was carried out by the dideoxy chain termination method of Sanger et al. (1977, Proceedings of the National Academies of Sciences, U.S.A., 74:5463-5467) with modifications according to Zimmermann et al. (1990, Nucleic Acids Research, 18:1067). 10 The "RR dRhodamin Terminator Cycle Sequencing Kit" from PE Applied Biosystems (Product No. 403044, Weiterstadt, Germany) was used. The separation by gel electrophoresis and analysis of the sequencing reaction were carried out in a "Rotiphoresis NF Acrylamide/Bisacrylamide" Gel (29:1) 15 (Product No. A124.1, Roth, Karlsruhe, Germany) with the "ABI Prism 377" sequencer from PE Applied Biosystems (Weiterstadt, Germany).

The raw sequence data obtained were then processed using the Staden program package (1986, Nucleic Acids Research, 20 14:217-231) version 97-0. The individual sequences of the pZerol derivatives were assembled to a continuous contig. The computer-assisted coding region analysis were prepared with the XNIP program (Staden, 1986, Nucleic Acids Research, 14:217-231). Further analyses were carried out 25 with the "BLAST search program" (Altschul et al., 1997, Nucleic Acids Research, 25:3389-3402) against the non-redundant databank of the "National Center for Biotechnology Information" (NCBI, Bethesda, MD, USA).

The resulting nucleotide sequence is shown in SEQ ID No. 1. 30 Analysis of the nucleotide sequence showed an open reading frame of 1875 bp, which was called the dead gene. The dead gene codes for a polypeptide of 624 amino acids.

## SEQUENCE PROTOCOL

<110> Degussa-Hüls AG

5 <120> New nucleotide sequences which code for the dead gene

<130> 000557 BT

<140>

10 <141>

<160> 2

<170> PatentIn Ver. 2.1

15

 $\langle 210 \rangle$  1

<211> 2381

<212> DNA

<213> Corynebacterium glutamicum

20

 $\langle 220 \rangle$ 

<221> CDS

$\langle 222 \rangle \quad (259) \dots (2130)$

<223> deaD gene

25

<400> 1

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actgttgaag atgcaccagc atctgagggt agcgaagaga tcaccagggt tgcggataact 120

30

tctgaggacg ccgactctgc agatgcagac aacgcgagca atgtaatcaa tgagaatgag 180

gactcctcgg aaggtgctaa ccagccttca aacgagtcac cctctacgga agccaaatcc 240

35

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1 5 10

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Val Gly Tyr Glu Thr Pro Ser Pro Ile Gln Ala Gln Thr Ile Pro Ile  
15 20 25

40

ctc atg gag ggc cag gat gtt gtt ggt cta gca cag acc ggt acc ggt 387  
Leu Met Glu Gly Gln Asp Val Val Gly Leu Ala Gln Thr Gly Thr Gly  
30 35 40

45

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Lys Thr Ala Ala Phe Ala Leu Pro Ile Leu Ala Arg Ile Asp Lys Ser  
45 50 55

50

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55

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80                      85                      90

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	Ser Tyr Val His Arg Ile Gly Arg Thr Gly Arg Ala Gly Arg Thr Gly	
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Gly Ala His Ile Val Val Gly Thr Pro Gly Arg Ile Ile Asp His Leu  
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5	Glu	Ala	Asp	Glu	Met	Leu	Asn	Met	Gly	Phe	Gln	Glu	Asp	Val	Glu	Arg	
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20		225				230					235					240	
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40	Val	Thr	Pro	Arg	Glu	Arg	Arg	Met	Leu	Arg	Ser	Ile	Glu	Arg	Ala	Thr	
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		370					375					380					
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 610 615 620

**Patent claims**

1. An isolated polynucleotide from coryneform bacteria,  
comprising a polynucleotide sequence which codes for  
5 the *dead* gene, chosen from the group consisting of
  - a) polynucleotide which is identical to the extent of  
at least 70% to a polynucleotide which codes for a  
polypeptide which comprises the amino acid sequence  
of SEQ ID No. 2,
  - 10 b) polynucleotide which codes for a polypeptide which  
comprises an amino acid sequence which is identical  
to the extent of at least 70% to the amino acid  
sequence of SEQ ID No. 2,
  - c) polynucleotide which is complementary to the  
15 polynucleotides of a) or b), and
  - d) polynucleotide comprising at least 15 successive  
nucleotides of the polynucleotide sequence of a),  
b) or c),

the polypeptide preferably having the activity of  
20 DNA/RNA helicase.
2. The polynucleotide as claimed in claim 1, wherein the  
polynucleotide is a preferably recombinant DNA which is  
capable of replication in coryneform bacteria.
3. The polynucleotide as claimed in claim 1, wherein the  
25 polynucleotide is an RNA.
4. The polynucleotide as claimed in claim 2, comprising  
the nucleic acid sequence as shown in SEQ ID No. 1.
5. The DNA as claimed in claim 2 which is capable of  
replication, comprising

- (i) the nucleotide sequence shown in SEQ ID No. 1,  
or
  - (ii) at least one sequence which corresponds to  
sequence (i) within the range of the  
degeneration of the genetic code, or
  - (iii) at least one sequence which hybridizes with the  
sequence complementary to sequence (i) or (ii),  
and optionally
  - (iv) sense mutations of neutral function in (i).
6. The DNA as claimed in claim 5 which is capable of  
replication, wherein the hybridization is carried out  
under a stringency corresponding to at most 2x SSC.
7. The polynucleotide sequence as claimed in claim 1,  
which codes for a polypeptide which comprises the amino  
acid sequence shown in SEQ ID No. 2.
8. Coryneform bacteria in which the *deadD* gene is  
attenuated, in particular eliminated.
9. A process for the fermentative preparation of L-amino  
acids, in particular L-lysine, wherein the following  
steps are carried out:
- a) fermentation of the coryneform bacteria which  
produce the desired L-amino acid and in which at  
least the *deadD* gene or nucleotide sequences which  
code for it are attenuated, in particular  
eliminated;
  - b) concentration of the L-amino acid in the medium or  
in the cells of the bacteria, and
  - c) isolation of the L-amino acid.

10. The process as claimed in claim 9, wherein bacteria in which further genes of the biosynthesis pathway of the desired L-amino acid are additionally enhanced are employed.
- 5 11. The process as claimed in claim 9, wherein bacteria in which the metabolic pathways which reduce the formation of the desired L-amino acid are at least partly eliminated are employed.
- 10 12. The process as claimed in claim 9, wherein the expression of the polynucleotide(s) which code(s) for the *deaD* gene is attenuated, in particular eliminated.
13. The process as claimed in claim 9, wherein the catalytic properties of the polypeptide (enzyme protein) for which the polynucleotide *deaD* codes are reduced.
- 15 14. The process as claimed in claim 9, wherein for the preparation of L-amino acids, coryneform microorganisms in which at the same time one or more of the genes chosen from the group consisting of
- 20 14.1 the *dapA* gene which codes for dihydrodipicolinate synthase,
- 14.2 the *gap* gene which codes for glyceraldehyde 3-phosphate dehydrogenase,
- 14.3 the *tpi* gene which codes for triose phosphate isomerase,
- 25 14.4 the *pgk* gene which codes for 3-phosphoglycerate kinase,
- 14.5 the *zwf* gene which codes for glucose 6-phosphate dehydrogenase,

- 14.6 the pyc gene which codes for pyruvate carboxylase,
- 14.7 the mgo gene which codes for malate-quinone oxidoreductase,
- 5 14.8 the lysC gene which codes for a feed-back resistant aspartate kinase,
- 14.9 the lysE gene which codes for lysine export,
- 14.10 the hom gene which codes for homoserine dehydrogenase
- 10 14.11 the ilvA gene which codes for threonine dehydratase or the ilvA(Fbr) allele which codes for a feed back resistant threonine dehydratase,
- 14.12 the ilvBN gene which codes for acetohydroxy-  
15 acid synthase,
- 14.13 the ilvD gene which codes for dihydroxy-acid dehydratase,
- 14.14 the zwal gene which codes for the Zwal protein
- 20 is or are enhanced or over-expressed are fermented.
- 15. The process as claimed in claim 9, wherein for the preparation of L-amino acids, coryneform microorganisms in which at the same time one or more of the genes  
25 chosen from the group consisting of
  - 15.1 the pck gene which codes for phosphoenol pyruvate carboxykinase,
  - 15.2 the pgi gene which codes for glucose 6-phosphate isomerase,



15.3 the poxB gene which codes for pyruvate oxidase

15.4 the zwa2 gene which codes for the Zwa2 protein

is or are attenuated are fermented.

- 5 16. Coryneform bacteria which contain a vector which carries parts of the polynucleotide as claimed in claim 1, but at least 15 successive nucleotides of the sequence claimed.
- 10 17. The process as claimed in one or more of the preceding claims, wherein microorganisms of the species *Corynebacterium glutamicum* are employed.
- 15 18. A process for discovering RNA, cDNA and DNA in order to isolate nucleic acids or polynucleotides or genes which code for DNA/RNA helicase or have a high similarity with the sequence of the *dead* gene, wherein the polynucleotide comprising the polynucleotide sequences as claimed in claims 1, 2, 3 or 4 is employed as hybridization probes.

**Abstract**

The invention relates to an isolated polynucleotide comprising a polynucleotide sequence chosen from the group  
5 consisting of

- a) polynucleotide which is identical to the extent of at least 70% to a polynucleotide which codes for a polypeptide which comprises the amino acid sequence of SEQ ID No. 2,
- 10 b) polynucleotide which codes for a polypeptide which comprises an amino acid sequence which is identical to the extent of at least 70% to the amino acid sequence of SEQ ID No. 2,
- c) polynucleotide which is complementary to the  
15 polynucleotides of a) or b), and
- d) polynucleotide comprising at least 15 successive nucleotides of the polynucleotide sequence of a), b) or c),

and a process for the fermentative preparation of L-amino  
20 acids using coryneform bacteria in which at least the dead gene is present in attenuated form, and the use of polynucleotides which comprise the sequences according to the invention as hybridization probes.